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E2F4/5 and p107 as Smad Cofactors Linking the TGFβ Receptor to c-*myc* Repression

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Summary

Smad3 is a direct mediator of transcriptional activation by the TGFβ receptor. Its target genes in epithelial cells include cyclin-dependent kinase inhibitors that generate a cytostatic reponse. We defined how, in the same context, Smad3 can also mediate transcriptional repression of the growth-promoting gene c-*myc*. A complex containing Smad3, the transcription factors E2F4/5 and DP1, and the corepressor p107 preexists in the cytoplasm. In response to TGFβ, this complex moves into the nucleus and associates with Smad4, recognizing a composite Smad-E2F site on c-*myc* for repression. Previously known as the ultimate recipients of cdk regulatory signals, E2F4/5 and p107 act here as transducers of TGFβ receptor signals upstream of cdk. Smad proteins therefore mediate transcriptional activation or repression depending on their associated partners.

Introduction

Growth factor signals control the expression of large ensembles of target genes through immediate effects on transcriptional activation as well as repression. TGFβ signals mediated by Smad transcription factors are a case in point. Of several hundred immediate gene responses triggered by TGFβ in a given cell, over half result in activation of the target and the rest result in repression (Chen et al., 2001; Zavadil et al., 2001). This raises the question of how a particular Smad complex moving into the nucleus in response to TGFβ stimulation finds its way to the appropriate gene promoter and identifies it as a predetermined target of activation or repression.

This question presented itself in the analysis of the TGFβ cytostatic program. TGFβ fosters tissue growth and morphogenesis during embryonic development (Bottinger et al., 1997). In the adult, however, TGFβ delivers cytostatic signals that help maintain tissue homeostasis (Massagué et al., 2000). Epithelial cells of the skin, lung, liver, gut, pancreas, breast, prostate, and ovary, as well as hematopoietic, lymphoid, and endothelial cells, are growth inhibited by TGFβ. Loss of responsiveness to TGFβ is a hallmark of many types of cancer, occurring through mutational inactivation of TGFβ receptors or Smads as well as selective losses of cytostatic gene responses (Chen et al., 2001; Derynck et al., 2001; Massagué et al., 2000).

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The TGFβ cytostatic program involves two classes of immediate gene responses (Massagué et al., 2000). One includes activation of genes encoding the cyclin-dependent kinase (cdk) inhibitors p15Ink4b and p21Cip1/Waf1 (Figure 1A). In combination with another cdk inhibitor, p27Kip1, mobilized from cdk4 bound storage, the induction of these proteins by TGFβ neutralizes the activity of cdk2 and cdk4 during G1 phase of the cell cycle. In the absence of this activity, the cdk substrates pRb, p107, and p130 remain bound to E2F transcription factors, preventing expression of E2F1-3 target genes (Classon and Dyson, 2001; Mulligan and Jacks, 1998; Trimarchi and Lees, 2002). As these genes encode activities required for DNA synthesis, their inhibition prevents cell cycle progression.

The other class of cytostatic TGFβ responses includes repression of growth-promoting transcription factors, most notably Myc (Alexandrow and Moses, 1995; Massagué et al., 2000). In addition to acting as a transcriptional activator of growth and proliferation (Eisenman, 2001; Grandori et al., 2000), Myc also acts as a direct inhibitor of *p15Ink4b* (Warner et al., 1999) and *p21Cip1/Waf1* (Claassen and Hann, 2000). Thus, transcriptional repression of c-*myc* in response to TGFβ removes growth-promoting functions while facilitating the induction of cdk inhibitors (Figure 1A; Seoane et al., 2001).

TGFβ controls gene expression from the cell surface by assembling a receptor serine/threonine kinase complex comprising subunits known as the type I receptor and type II receptor (Heldin et al., 1997; Massagué, 1998). Ligand-induced phosphorylation by the type II receptor enables the type I receptor to recognize and phosphorylate Smad2 and Smad3. Phosphorylation releases these proteins from cytoplasmic retention, allowing their translocation into the nucleus and association with a related factor, Smad4. This assembly forms the core of transcriptional regulatory complexes.

Work on genes that are activated by TGFβ or the related factors activin, nodal, and bone morphogenetic proteins (BMP) have revealed that an activated Smad complex recognizes regulatory elements in target genes with the help of associated DNA binding cofactors (Derynck et al., 1998; Massagué and Wotton, 2000; Whitman, 1998). Members of different families of transcription factors have been recently shown to function as Smad partners in this fashion. Combined, the DNA binding ability of Smads and their partners provide each particular complex with high affinity and specificity toward a particular target gene.

Little is known about what takes place between the recognition of a particular gene by a Smad complex and the decision of whether to activate or repress that gene. Smads can directly bind the transcriptional coactivators p300, CBP, and Smif, which are thought to mediate activation of at least some TGFβ target genes (Bai et al., 2002; Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998; Shen et al., 1998). Smads can interact with transcriptional corepressors as well, including TGIF (Wotton et al., 1999) and the related proteins Ski and SnoN (Luo et al., 1999; Sun et al., 1999). So far,

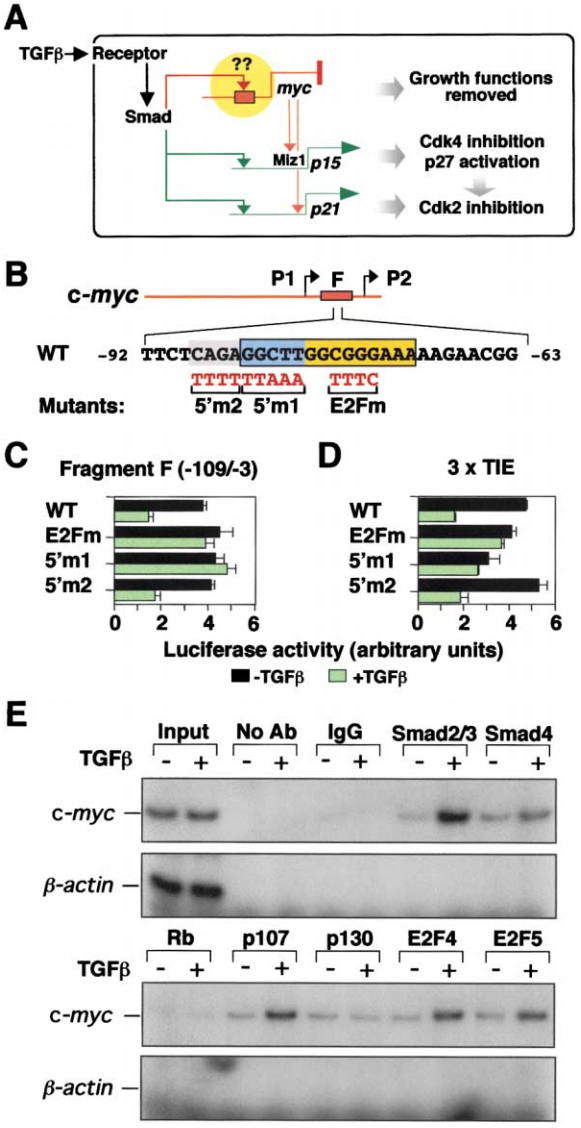

Figure 1. Role of Adjacent Smad and E2F Sites and Rapid Binding of Smads, E2F4/5, and p107 to the c-*myc* Promoter in Response to TGFβ

(A) Schematic representation of a TGFβ cytostatic program.

(B) Schematic representation of the human and rat c-*myc* promoter and nucleotide sequence of the TIE element. P1 and P2 indicate the transcription start sites, and the box marks the position of fragment F used in luciferase reporter assays. Nucleotide sequence positions are indicated relative to the P2 transcription start site. The E2F site (yellow), the putative Smad binding site (blue), and the mutations targeting these sites and an upstream sequence are indicated.

(C and D) HaCaT cells were transfected with the indicated wild-type (wt) or mutant TIE reporter constructs, treated with or without TGFβ for 16–20 hr prior to lysis, and analyzed for luciferase activity. Reporter constructs used include pFrag-F, pFrag-F-E2Fm, pFrag-F-5′m1, pFrag-F-5′m2, p3xTIE, 3xTIE-E2Fm, p3xTIE5′m1, and p3xTIE5′m2.

(E) HaCaT cells were left untreated (−) or treated (+) with TGFβ for 1 hr, chromatin immunoprecipitations were performed with the indicated antibodies, and PCR was performed with primers specific for the c-*myc* promoter region harboring the TIE and primers specific for the β-actin promoter as TGFβ unresponsive control.

however, these corepressors have been shown to constrain the basal activity of Smad complexes rather than mediate TGFβ-dependent gene repression responses.

Thus, the problem of how target gene repression is decided over activation incorporates several long-standing questions about TGFβ action and its dependence on cell type, signaling context, and target gene. These issues remain unsolved for many other growth and differentiation factors as well. The present identification of E2F4/5 and p107 as signal cotransducers that enable a Smad complex to recognize and repress c-*myc* sheds light on some of these questions.

Results

Adjacent Smad and E2F Sites in c-*myc* Mediate Repression by TGFβ

A TGFβ inhibitory element (TIE) located between positions −92 and −63 relative to the P2 transcription start site of c-*myc* mediates transcriptional repression in response to TGFβ in human skin keratinocytes and mammary epithelial cells (Figure 1B; Chen et al., 2001). Used as a biotinylated double-stranded oligonucleotide probe, this TIE recognizes a Smad3-Smad4 complex in extracts from TGFβ-treated cell lines (Chen et al., 2001; Yagi et al., 2002). We set out to identify the origin of this complex and the components that enable it to selectively bind to the TIE and mediate repression.

Mutational analysis of the TIE and surrounding region did not reveal any canonical Smad binding site (GTCT sequence) essential for the TGFβ response. However, the TIE encompasses a canonical E2F site (Figure 1B; Chen et al., 2001; Yagi et al., 2002). This site is flanked 5′ by a sequence GGCT. Although this is an imperfect Smad contact site, the Smad3-GTCT interaction admits substitutions in the second base (Shi et al., 1998). We investigated the role of these sites in HaCaT human keratinocytes, a spontaneously immortalized cell line that retains many properties of normal counterparts (Boukamp et al., 1988) including a cytostatic response to TGFβ (Reynisdóttir et al., 1995). Wild-type and mutant versions of the –109/–3 region (fragment F) of c-*myc* were tested as transcriptional reporter constructs in HaCaT cells (Figure 1C). Mutations targeting the GGCT sequence or the E2F site abolished the TGFβ response. Mutations upstream of this sequence had no effect. The effects of these mutations were similar when tested in the context of a construct containing three repeats of the TIE (Figure 1D). Thus, the integrity of the E2F site is as important as that of the Smad site for TGFβ-dependent repression from the c-*myc* TIE.

Rapid Binding of Smad3, Smad4, E2F4/5, and p107 to the c-*myc* Promoter in Response to TGFβ

We carried out chromatin immunoprecipitation (ChIP) assays to determine if Smad proteins bind to the c-*myc* promoter along with a corepressor E2F complex in response to TGFβ. Smad2 and Smad3 are direct substrates of the TGFβ type I receptor kinase, and their phosphorylation enables association with Smad4 and translocation into the nucleus. Smad2 and Smad3 are of nearly identical amino acid sequence except for the presence of a unique insert in Smad2 that prevents its

direct binding to DNA (Shi et al., 1998). ChIP assays using anti-Smad2/3 or anti-Smad4 antibodies demonstrated that Smad2/3 and Smad4 specifically bind to the proximal region of the c-*myc* promoter within 1 hr of TGFβ addition (Figure 1E).

The activity of E2F factors is determined by their association with transcriptional corepressors of the retinoblastoma family, pRb, p107, and p130 (Classon and Dyson, 2001; Mulligan and Jacks, 1998; Trimarchi and Lees, 2002). This association is inhibited by cdk-mediated phosphorylation of pRb, p107, and p130. E2F1, E2F2, and E2F3 function primarily as transcriptional activators that become inhibited by association with pRB. In contrast, E2F4 and E2F5 act as transcriptional repressors in association with p107 or p130. ChIP assays using suitable antibodies demonstrated that p107, E2F4, and E2F5 specifically bind to the c-*myc* promoter in response to TGFβ (Figure 1E), whereas pRb, p130, and E2F1-3 do not (Figure 1E and data not shown). Thus, TGFβ induces rapid binding of Smad2/3, Smad4, E2F4/5, and p107 to the c-*myc* promoter in vivo.

The c-*myc* TIE Accommodates Smad3-Smad4 and E2F4-DP Complexes

To determine if binding of these proteins to the c-*myc* promoter occurs in the TIE region, we conducted DNA precipitation (DNAP) assays using biotinylated double-stranded TIE oligonucleotides. The wild-type TIE probe precipitated endogenous Smad3 and Smad4 from TGFβ-treated cells but not control cells (Figure 2A). Smad2 was more abundant than Smad3 in the cells but was present only in trace amounts in the TIE DNA precipitates (Figure 2A, compare first and third lanes). Thus, the TIE recognizes a Smad3-Smad4 complex in preference over a Smad2-Smad4 complex. This is consistent with the observation that skin keratinocytes from *Smad3*$^{-/-}$ mice are defective in cytostatic but not other TGFβ responses (Ashcroft et al., 1999).

Smad binding to the TIE in the DNAP assays was diminished by mutations targeting the E2F site (Figure 2A, E2Fm lane) as effectively as it was by mutations targeting the Smad site (Figure 2A, 5′m1 lane). When directly used on cell lysates, the TIE oligonucleotide precipitated different E2F proteins indiscriminately and in a TGFβ-independent manner (data not shown). This is not surprising because E2F-DP in a cell-free system can unrestrictedly bind to consensus E2F sites (Buck et al., 1995; Lees et al., 1993; Zhang and Chellappan, 1995). However, when we first immunoprecipitated Smad3 from a transfected COS cell extract, resuspended this precipitate, and subjected the sample to TIE DNAP, we observed a TGFβ-dependent and -specific interaction with E2F4 (Figure 2B). E2F family members bind to E2F sites as heterodimers with DP1 or DP2 (Trimarchi and Lees, 2002). We observed a TGFβ-dependent association of DP1 and p107 in the Smad-containing TIE precipitates (Figure 2B). These results suggest that a complex containing Smad3, E2F4, DP1, and p107 binds rapidly to the c-*myc* TIE in response to TGFβ.

To see if simultaneous binding of Smad and E2F to the c-*myc* TIE is feasible from a structural standpoint, the crystal structures of the Smad3 DNA binding domain bound to GTCT (Shi et al., 1998) and the E2F4-DP2 DNA binding domains bound to an E2F site (Zheng et al., 1999) were oriented to match the disposition of the Smad and E2F sites on the c-*myc* TIE (Figure 2C). In the resulting model, the TIE can accommodate Smad3 and E2F4-DP2 without clashing with each other. In fact, these proteins sit sufficiently close to establish protein-protein interactions on the DNA. In particular, the region comprising residues 106–114 in the long α helix of DP2 is positioned close to the loop between α helices 2 and 3 and the loop preceding the DNA-contacting β hairpin of Smad3.

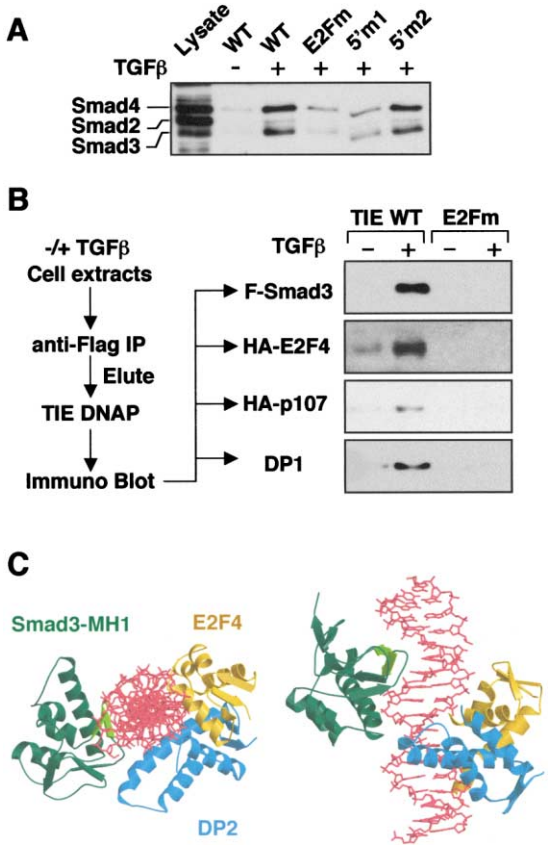

Figure 2. TGFβ-Induced Binding of Smad3-Smad4 and E2F4-DP to the c-*myc* TIE

(A) HaCaT cells were incubated in the absence (−) or presence (+) of TGFβ for 1 hr. Cell lysates were incubated with the indicated wild-type (wt) or mutant (E2Fm, 5′m1, and 5′m2) biotinylated double-stranded TIE oligonucleotides. DNA bound proteins, were precipitated by streptavidin-agarose and the presence of Smad2, Smad3, or Smad4 was detected by immunoblotting. Total HaCat cell extract (Lysate) was included as a reference.

(B) COS-1 cells were transfected with Flag-Smad3, HA-E2F4, and HA-p107 expression constructs. Cells were treated with (+) or without (−) TGFβ and subjected to sequential immunoprecipitation, DNA pull-down (DNAP), and immunoblotting analyses as diagrammed on the left. Wild-type (TIE WT) and mutant (E2Fm) biotinylated TIE oligonucleotides were used in the DNAP. The presence of Flag-Smad3, HA-E2F4, HA-p107, and DP1 in the DNAP was detected with anti-Flag, anti-HA, or anti-DP1 antibodies.

(C) The X-ray crystal structure of the Smad3 MH1 domain bound to GTCT (Shi et al., 1998) and the DNA binding domains of E2F4 and DP2 binding jointly to an E2F site (Zheng et al., 1999) were oriented to match the disposition of the Smad and E2F sites on the c-*myc* TIE. Orthogonal views of the resulting model are shown.

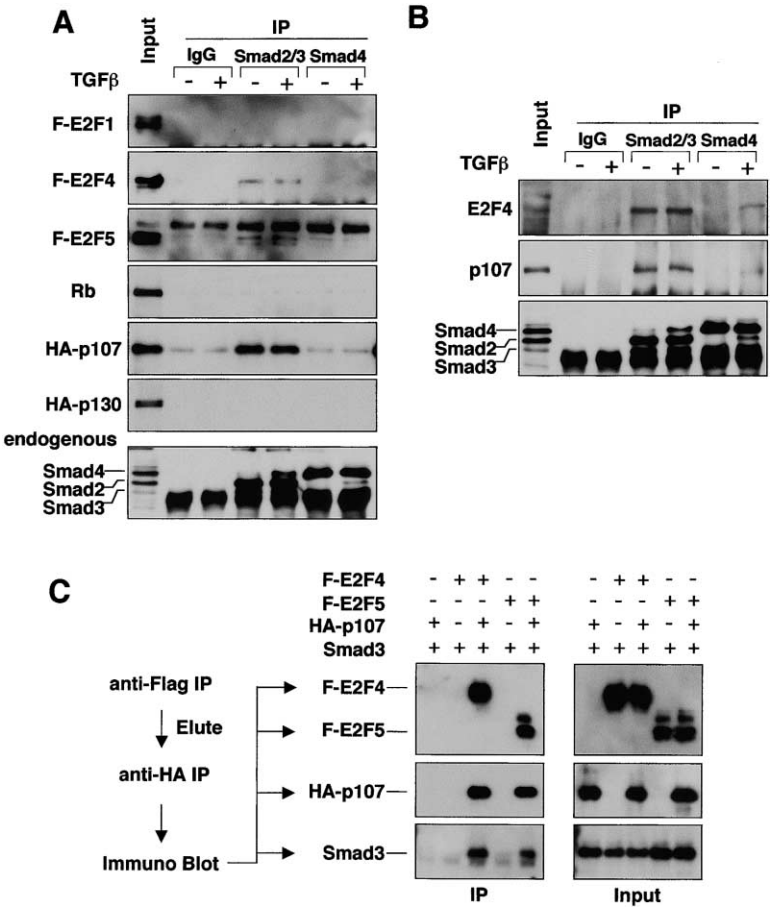

Figure 3. A Preexisting Smad3-E2F4/5-p107 Complex

(A) COS-1 cells were transfected with vectors encoding the indicated Flag epitope-tagged E2F proteins, HA epitope-tagged p107 and p130, or nontagged Rb. The cells were treated with (+) or without (−) TGFβ for 1 hr, and lysates were precipitated with the indicated anti-Smad antibodies or purified rabbit IgG as a control. The immunoprecipitates were subjected to immunoblotting with antibodies specific for pRb or the indicated epitope tags. Protein expression was assessed by direct immunoblotting of total lysates (Input).

(B) HaCaT cells were treated with (+) or without (−) TGFβ for 1 hr prior to lysis, and immunoprecipitations were performed with the indicated antibodies. Smad-associated E2F4 and p107 were detected by immunoblot analysis.

(C) COS-1 cells were transfected with vectors encoding HA-p107, Flag-E2F4, Flag-E2F5, and/or Smad3 as indicated. Lysates were subjected to immunoprecipitation with anti-Flag antibodies. Immune complexes were eluted and subjected to immunoprecipitation with anti-HA antibody. The latter precipitate was subjected to immunoblotting with anti-HA to detect HA-p107, anti-Flag to detect F-E2F4 or F-E2F5, and anti-Smad2/3 antiserum to detect Smad3. The expression of transfected proteins was monitored by immunoblotting of total cell extracts (Input).

A Preexisting Smad3-E2F4/5-p107 Complex

To investigate the physical interactions of Smads with E2F and pRb family members, we expressed epitope-tagged versions of these proteins in COS cells and assessed their ability to coimmunoprecipitate with endogenous Smads. E2F4, E2F5, and p107 specifically interacted with Smad2/3, whereas E2F1, pRb, and p130 did not, even when overexpressed (Figure 3A). TGFβ addition had no effect on these interactions.

We confirmed the ability of endogenous Smad proteins to interact with endogenous E2F4 and p107 by immunoprecipitation of HaCaT cell lysates with anti-Smad2/3 and anti-Smad4 antibodies followed by anti-E2F4 or anti-p107 immunoblotting (Figure 3B). The Smad2/3-p107 and Smad2/3-E2F4 interactions did not require cell stimulation with TGFβ, while the Smad4-p107 and Smad4-E2F interactions were solely observed in the presence of TGFβ. A sequential immunoprecipitation protocol showed that Smad3, flag-tagged E2F4 (or E2F5), and HA-tagged p107 can coexist in the same complex (Figure 3C). Collectively, these results indicate that cells contain a preassembled Smad3-E2F4-p107 complex, and Smad4 is incorporated into this complex on TGFβ stimulation.

Independent Smad3-p107 and Smad3-E2F4/5 Contacts

p107 binds directly to the C-terminal region of E2F4 and E2F5 (Classon and Dyson, 2001). To determine which component of the p107-E2F4/5 complex establishes an interaction with Smad3, we subjected these proteins and relevant deletion constructs to immunoprecipitation-immunoblotting analysis in transfected COS cells. In cells cotransfected with Smad3 and E2F4 or E2F5 vectors, the E2F proteins were detectable in Smad3 immunoprecipitates, and vice versa (Figures 4A, 4B, 4D, and 4E). Using deletion constructs, we mapped the Smad-interacting region to the N-terminal segment of E2F4 (residues 1–99) and E2F5 (residues 1–132) (Figures 4D and 4E). This region includes the DNA binding domain, which forms a tight fold (Zheng et al., 1999), and a short segment that connects with the DP dimerization domain, which falls outside this region. The DNA binding domain is highly conserved among E2F family members, but contains small clusters of residues unique to E2F4 and E2F5. Because Smad3 does not interact with E2F1, we surmise that these clusters determine the specificity of the Smad3-E2F4/5 interaction.

Using Smad3 deletion constructs, we mapped the E2F4/5 interacting region to the MH2 domain of Smad3 (Figures 4A and 4B). The MH2 domain is a tightly packed globular structure that also binds to the type I TGFβ receptor (Huse et al., 2001; Lo et al., 1998), the Smad anchor for receptor activation SARA (Tsukazaki et al., 1998; Wu et al., 2000), partner Smads (Qin et al., 2001; Shi et al., 1997; Wu et al., 2001a), DNA binding cofactors (Chen et al., 1998; Randall et al., 2002), coactivators, and corepressors (Massagué and Wotton, 2000). X-ray

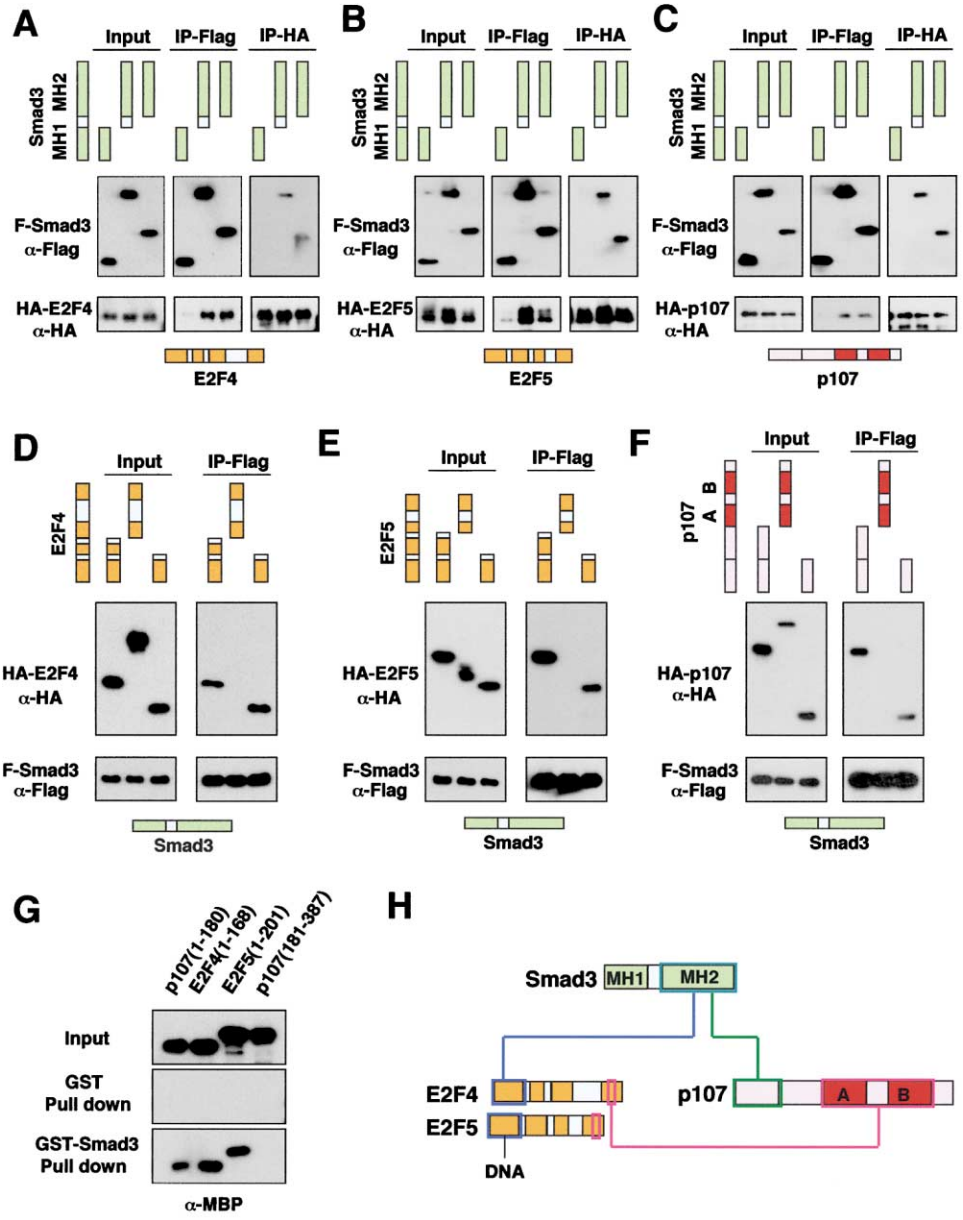

Figure 4. Smad3-p107 and Smad3-E2F4/5 Interaction Domains

(A–F) COS-1 cells were transfected with vectors encoding HA-E2F4, HA-E2F5, HA-p107, and/or Flag-Smad3 as indicated at the bottom of each panel, together with the deletion constructs diagrammed at the top of each panel. Cell lysates were divided and immunoprecipitated with the antibodies indicated at the top of each panel. Immunoprecipitates were analyzed by immunoblotting with the antibodies indicated on the left of each panel. Expression of the proteins was monitored by immunoblotting of total cell extracts (Input).

(G) GST control or GST-Smad3 bound to glutathione beads were mixed with purified maltose binding protein (MBP)-fusion proteins (indicated at top). MBP-fusion proteins bound to the beads were detected by immunoblotting with anti-MBP antibodies. The amount of each MBP-fusion protein used in the GST pull-downs was monitored by immunoblot analysis of total cell extracts (Input).

(H) A schematic representation of protein-protein interactions within the Smad3-p107-E2F4/5 complex. The interacting domains are interconnected by lines. Abbreviations: DNA, DNA binding domain of E2Fs; A and B, the two regions of the A/B domain.

crystal structures of Smad-Smad (Qin et al., 2001; Shi et al., 1997; Wu et al., 2001a) and Smad-SARA complexes (Wu et al., 2000) have revealed the involvement of distinct MH2 domain surfaces in these interactions, with an important contribution of the receptor-phosphorylated C-terminal tail to the interaction with Smad4 (Wu et al., 2001a).

We also detected an interaction between Smad3 and p107 (Figures 4C and 4F). Using deletion constructs, we mapped the interacting regions to the MH2 domain of Smad3 and the N-terminal 180 amino acid region of p107. This region is separate from the E2F-interacting region of p107, which corresponds to the A/B domain (Classon and Dyson, 2001). To determine if the Smad3-p107 and Smad3-E2F4/5 interactions are direct, we performed in vitro interaction assays using purified, recombinant pro-

teins (Figure 4G). A GST (glutathione S-transferase)-Smad3 fusion protein specifically bound the recombinant fragments p107(1–180), E2F4(1–168), and E2F5(1–201), but not the negative control p107(181–387). Therefore, Smad3 establishes direct, independent contacts with specific domains of p107 and E2F4/5. The three binary protein-protein interactions involved in the Smad3-p107-E2F4/5 complex are depicted in Figure 4H.

Cytoplasmic Smad3-E2F4/5-p107 Complex and Its TGFβ-Dependent Nuclear Translocation

Smad3 is accumulated in the cytoplasm until it becomes phosphorylated by the TGFβ receptor and moves into the nucleus (Heldin et al., 1997; Massagué, 1998). p107 is present in both compartments (Trimarchi and Lees, 2002). Unlike E2Fs 1–3, which are constitutively nuclear, E2F4 and E2F5 contain a nuclear localization signal and a CRM1-dependent nuclear export signal and are also present both in the cytoplasm and the nucleus (Gaubatz et al., 2000). Therefore, the subcellular distribution of these proteins is compatible with the notion that a Smad3-p107-E2F4/5 complex could reside in the cytoplasm awaiting TGFβ receptor-mediated activation. This was demonstrated by Smad2/3 immunoprecipitation from a HaCaT cytosolic fraction followed by anti-E2F4 or anti-p107 immunoblotting (Figure 5B). The same result was obtained using a DNase I-treated sample of the same cytosolic fractions (Figure 5B), eliminating the possibility that these protein interactions are bridged by contaminating DNA.

Quantitation of the endogenous p107 in HaCaT cell lysates and Smad2/3 immunoprecipitates revealed that only between 1.0% and 1.7% of the total p107 was bound to Smad2/3. Movement of such a small proportion into the nucleus in response to TGFβ would not significantly affect the overall distribution of p107 in the cell (Figure 5A). In order to visualize a TGFβ- and Smad3-dependent translocation of endogenous p107 into the nucleus, we overexpressed Smad3 in COS cells and assessed the distribution of the proteins by immunofluorescence. COS cells have the added advantage that their endogenous p107 is largely nuclear in contrast to the more even distribution seen in HaCaT cells (Figure 5A). In the absence of TGFβ, cells overexpressing Flag-tagged Smad3 showed a higher level of endogenous p107 in the cytoplasm than did their untransfected neighbors (Figure 5C). TGFβ addition reduced the level of cytoplasmic Flag-Smad3 and that of cytoplasmic p107 in the same cells (Figure 5C). Thus, a high level of Smad3 in the cytoplasm displaced endogenous p107 out of the nucleus, and TGFβ addition forced this p107 back into the nucleus.

Overexpression of the Smad binding region (N-terminal 180 amino acids) of p107 reduced the interaction between p107 and Smad3 with little effect on the E2F4-p107 interaction (Figure 5D). Overexpression of this construct inhibited the ability of Smad3 to force endogenous p107 out of the nucleus (Figure 5C), arguing that the observed redistribution of p107 is driven by its interaction with Smad3.

Mediation of the c-*myc* Response throughout the Cell Cycle

p107 is phosphorylated by cdk as the cell approaches S phase (Classon and Dyson, 2001). The hyperphosphorylated (electrophoretically slower) form of p107 is underrepresented in Smad2/3 complexes, as seen by comparing Smad2/3-associated p107 with total p107 (Figure 6A). These observations raised questions about the availability of p107-Smad3 complexes throughout the cell cycle and the ability of TGFβ to regulate c-*myc* during periods beyond G1 phase. To address these questions, we used MO-91 cells, a human acute myelogenous leukemia line that has a strong cytostatic response to TGFβ with downregulation of c-*myc*, as in epithelial cells (J. Scandura and S. Nimer, personal communication). MO-91 cells grow in suspension and therefore are suitable for centrifugal elutriation.

Asynchronous MO-91 cultures were elutriated into fractions enriched for cells in G1, S, or G2/M phase (Figure 6B, top). As judged by its electrophoretic mobility, p107 was largely in the underphosphorylated form in the G1 cells and accumulated as the hyperphosphorylated form in the S and G2/M cells (Figure 6B, p107). However, some underphosphorylated p107 was present throughout the cell cycle. Indeed, Smad2/3 was associated with underphosphorylated p107 during S phase (Figure 6C). Consistent with the persistent availability of underphosphorylated p107, incubation of the elutriated fractions with TGFβ for 2 hr resulted in Myc downregulation regardless of the cell cycle phase (Figure 6B).

Cell Cycle-Independent Repression of c-*myc*

In HaCaT cells, TGFβ induces the upregulation of cdk inhibitors p15Ink4b and p21Cip1/Waf1, which contributes to cdk inhibition and accumulation of underphosphorylated pRb (Reynisdóttir et al., 1995). This might secondarily lead to repression of c-*myc* along with many other genes (Ren et al., 2002). Therefore, we determined whether p15 and p21 induction and cdk inhibition play any role in the rapid downregulation of c-*myc* by TGFβ. Treatment of HaCaT cells with cycloheximide, a protein synthesis inhibitor, before TGFβ addition did not block c-*myc* downregulation by TGFβ (Figure 6D), suggesting that synthesis of cdk inhibitors is not required for this response. Next, we examined the kinetics of the c-*myc* response, the p15 and p21 induction, and the cdk inhibition in TGFβ-treated HaCaT cells. c-*myc* was downregulated within 1 hr of TGFβ addition, which is much faster than the accumulation of p15 or p21 or the progressive inhibition of cdk2 kinase activity in these cells (Figure 6E).

We also examined the effect of p15 induction and cdk2 inhibition on c-*myc* expression using a mink lung epithelial cell line Mv1Lu-tet-p15 that expresses ectopic p15 in a tetracycline-regulated fashion (Reynisdóttir and Massagué, 1997). Induction of ectopic p15 caused a progressive inhibition of cdk2 without downregulating c-*myc*, at least in the short term (first 12 hr; Figure 6F). In contrast, TGFβ addition to the same cells immediately led to c-*myc* downregulation. These data demonstrate that induction of cdk inhibitors or cdk inhibition per se do not mediate the rapid downregulation of c-*myc* seen in TGFβ-treated cells.

Requirement of E2F4/E2F5 and p107 for c-*myc* Repression by TGFβ

Several experiments were conducted to seek evidence that the Smad-p107-E2F4/5 complex is required for the c-*myc* response to TGFβ. Reporter assays using the

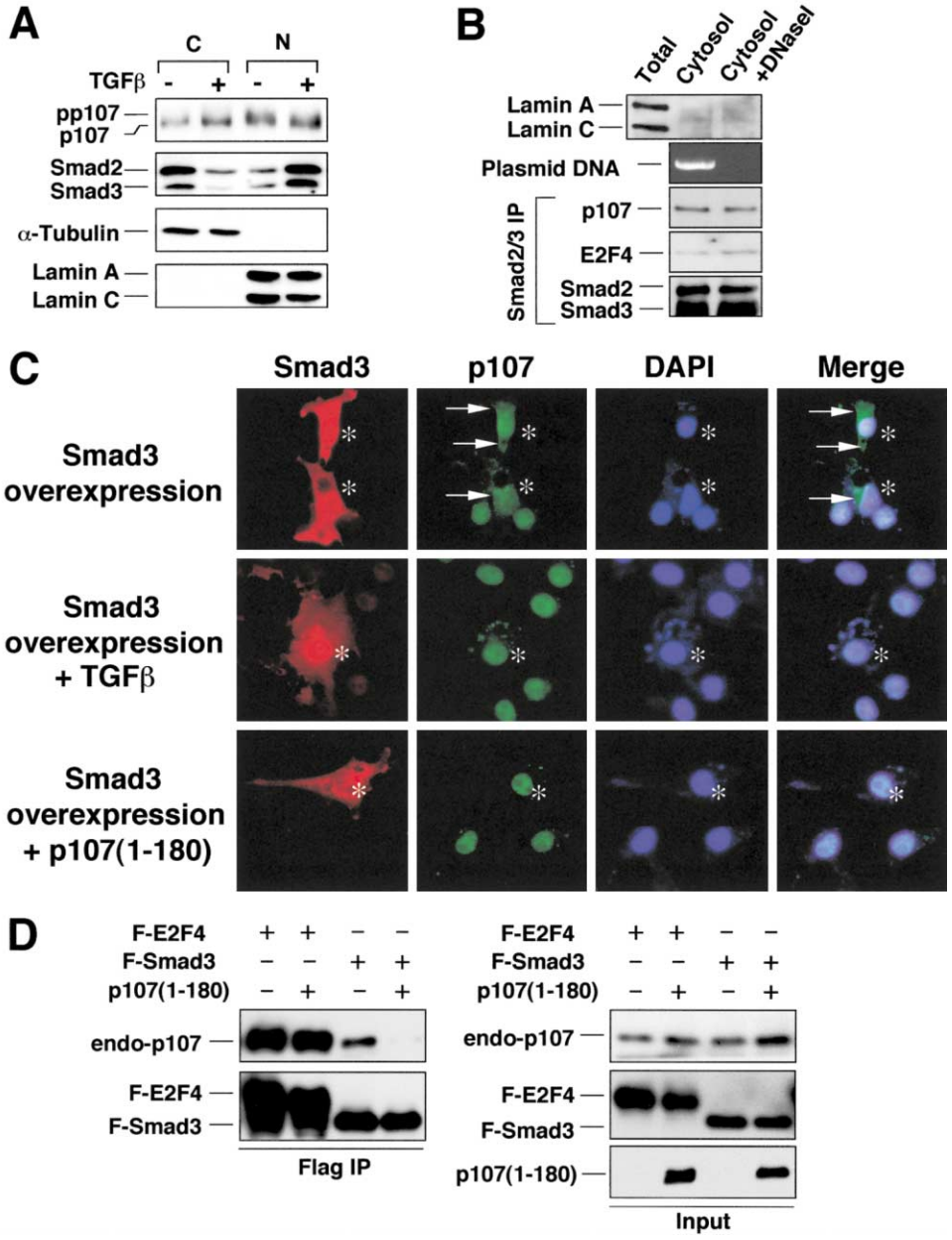

Figure 5. Subcellular Localization and Nuclear Translocation of p107 and Smad2/3

(A) HaCaT cells were incubated for 1 hr in the presence (+) or absence (−) of TGFβ (100 pM). Cytosolic (C) and nuclear fractions (N) were prepared and subjected to immunoblot analysis with anti-p107 and anti-Smad2/3 antibodies. Immunoblotting for α-tubulin and lamin A/C served as markers for cytoplasmic and nuclear fractions, respectively. The underphosphorylated (p107) and hyperphosphorylated (pp107) forms of p107 are indicated.

(B) Cytosolic fraction was prepared from HaCaT cells and treated with or without DNaseI prior to Smad2/3 immunoprecipitation. Smad-associated p107 and E2F4 were detected by immunoblotting. Immunoblot analysis of lamin A/C controlled for the purity of the cytosolic fraction when compared to total cell lysate (Total). Plasmid DNA was added to lysates to monitor the efficiency of DNAaseI digestion.

(C) COS-1 cell cultures were transfected with or without the indicated vectors. Two days after transfection, cells were incubated in the presence (+) or absence (−) of TGFβ for 1 hr and then subjected to immunofluorescence analysis with affinity-purified anti-Smad2/3 or anti-p107 antibodies to localize transfected Smad3 and endogenous p107, respectively. Nuclei were visualized by DNA staining with DAPI.

(D) COS-1 cells were transfected with vectors encoding Flag-E2F4, Flag-Smad3, and/or HA-p107(1–180), as indicated. Lysates were subjected to immunoprecipitation with an anti-Flag antibody. The E2F4 or Smad3-associated endogenous p107 (endo-p107) was detected by immunoblot analysis. The expression of transfected proteins was monitored by immunoblotting of total cell extracts (Input).

Smad-interacting regions of p107 and E2F4 as dominant-negative constructs showed that these constructs attenuated the downregulation of the c-*myc* promoter, whereas the homologous region of E2F1 had no effect (Figure 7A). Furthermore, TGFβ treatment caused a rapid decrease in c-*myc* mRNA levels in T cells from wild-type and *p130*$^{-/-}$ mice but not T cells from *p107*$^{-/-}$ mice (Figure 7B). siRNA-mediated depletion of *E2f4* or *E2f5*

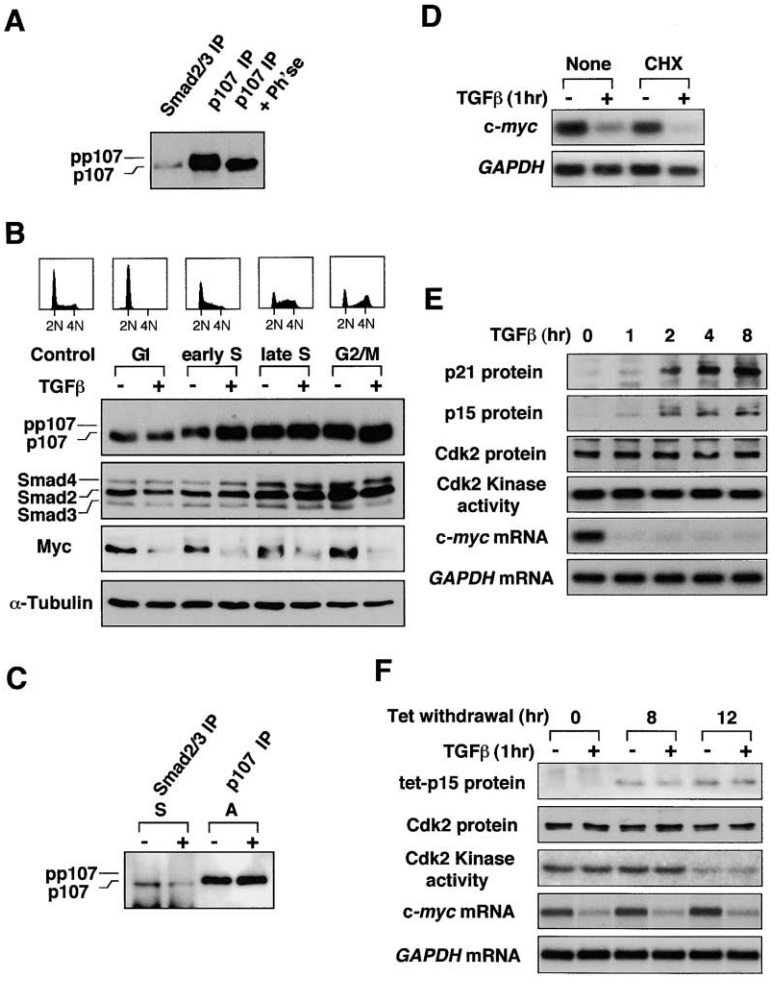

Figure 6. Smad3 Association with p107 and Mediation of the c-*myc* Response throughout the Cell Cycle

(A) Lysates from TGFβ-stimulated HaCaT cells were immunoprecipitated with anti-Smad2/3 or anti-p107 antibodies as indicated. The immunoprecipitates were left untreated or treated with protein phosphatase, and the presence of p107 was detected by immunoblotting with anti-p107 antibodies. The underphosphorylated (p107) and hyperphosphorylated (pp107) forms of p107 are indicated.

(B) Exponentially growing MO-91 cells were subjected to elutriation. The cell cycle distribution was determined by flow cytometry of samples from each fraction and from a control unfractionated culture (top). The fractions were left untreated or treated with TGFβ for 2 hr and then subjected to immunoblotting with the antibodies indicated on the left.

(C) Samples from the S phase fractions shown in (B) were subjected to anti-Smad2/3 immunoprecipitation followed by anti-p107 Western immunoblotting. p107 precipitates from asynchronous MO-91 cultures were used as a source of underphosphorylated (p107) and hyperphosphorylated (pp107) markers.

(D) HaCaT cells were treated with or without 10 μg/ml cycloheximide (CHX) for 1 hr and subsequently with (+) or without (−) TGFβ for an additional 1 hr. Total RNA was isolated, and the RNA level of c-*myc* and *GAPDH* were detected by Northern blot analysis.

(E) HaCaT cells were treated with TGFβ for the indicated times. Lysates and total RNA were prepared and the protein level of p15, p21, and Cdk2 and the RNA level of c-*myc* and *GAPDH* were determined by immunoblotting and Northern blot. Cdk2-associated kinase activity was examined in Cdk2 immunoprecipitates using a GST-Rb fusion protein as a substrate.

(F) Mv1Lu cells engineered to conditionally overexpress p15 in the absence of tetracycline were withdrawal from tetracycline inhibition for the indicated times and treated with (+) or without (−) TGFβ for an additional 1 hr. The protein level of p15 and Cdk2, the RNA level of c-*myc* and *GAPDH*, and the Cdk2-associated pRb kinase activity were determined as in (E).

messages in HaCaT cells had little or no effect on the c-*myc* response to TGFβ. When combined, however, the *E2f4* and *E2f5* siRNAs caused a marked inhibition of this response (Figure 7C). Furthermore, cell treatment with *E2f4* and *E2f5* siRNA inhibited the Smad2/3-p107 interaction (Figure 7D). These results provide strong genetic evidence for the importance and specificity of p107, E2F4/5, and their contacts with Smad3 in the c-*myc* response to TGFβ.

Discussion

Current models of TGFβ action envision that receptor-activated Smad proteins move into the nucleus, recruit transcriptional coactivators, and associate with a host of DNA binding cofactors, generating different transcriptional complexes, each with high affinity and specificity toward a particular gene or set of genes (Massagué et al., 2000; Whitman, 1998). Several such complexes have been identified and shown to mediate transcriptional activation of important genes. This model, however, sidesteps the question of how transcriptional repression of other genes is executed by the same mediators in the same cells. Transcriptomic analyses reveal that many immediate TGFβ gene responses are, in fact, repression responses (Chen et al., 2001; Zavadil et al., 2001). Among these, repression of c-*myc* has long been known as part of the cytostatic effect of TGFβ (Alexandrow and Moses, 1995). Thus, questions arise about the basis for the choice of repression over activation when Smads bind to a target gene.

The present work addresses some of these questions. By identifying E2F4/5 and DP1 as the DNA binding partners of Smad3 and Smad4 in c-*myc* promoter recognition and p107 as the corepressor jointly recruited by these proteins, the present results delineate a process of gene repression by TGFβ.

A Role for E2F4/5 and p107 as Cotransducers of TGFβ Signals

The role of E2F4/5 and p107 as cotransducers of TGFβ receptor signals suggested by our results is in contrast

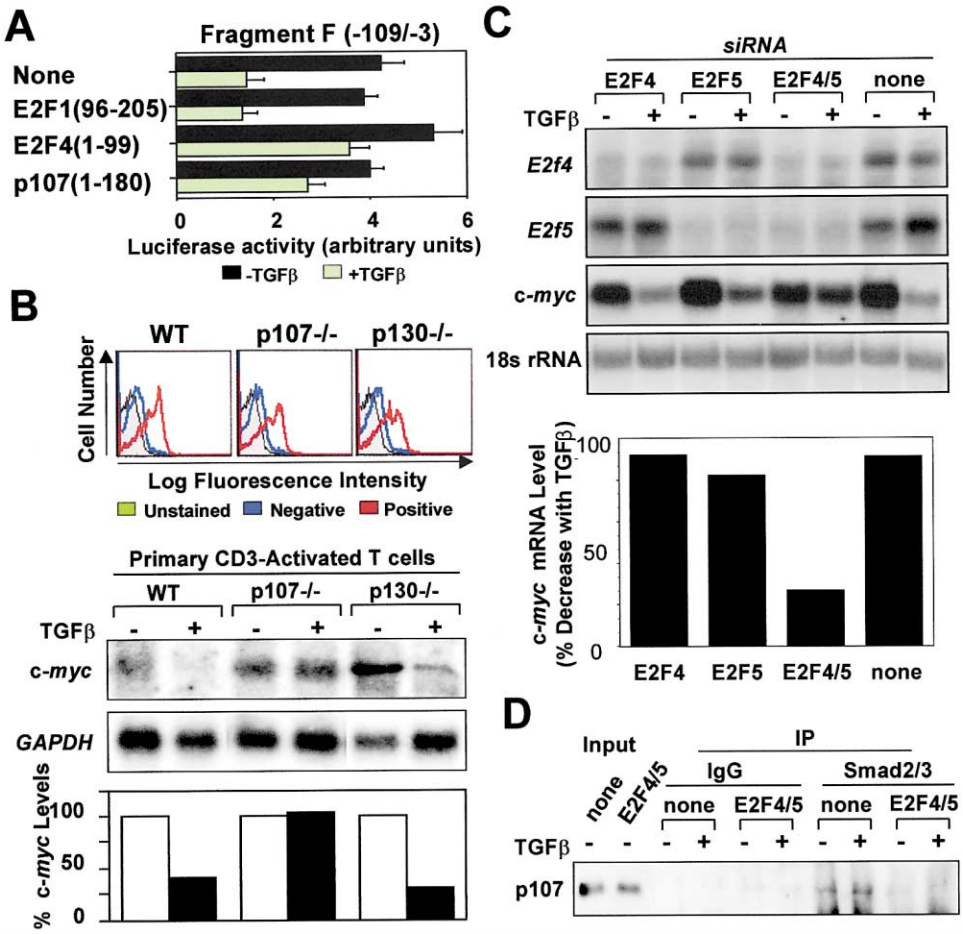

Figure 7. Requirement of E2F4 and E2F5 for the Downregulation of c-*myc* Transcription by TGFβ

(A) HaCaT cells were transfected with the indicated reporter and expression constructs, treated with (+) or without (−) TGFβ, and analyzed for luciferase activity. Constructs used were pFrag-F, pHA-E2F1(96–205), pHA-E2F4(1–99), and pHA-p107(1–180).

(B) Positive selection of mouse $CD90^{+}$(Thy 1.2) T cells was assessed by FACs analysis using anti-CD3 antibody. The positive fraction from wt, $p107^{-/-}$, and $p130^{-/-}$ was found to contain 70%, 63%, and 61% CD3-positive T cells, respectively. T cells were cultured for 48 hr in wells coated with 1.0 μg/ml of anti-mouse CD3 antibody to facilitate activation and were incubated for an additional 2 hr in the absence (−) or presence (+) of TGFβ (100 pM). The levels of c-*myc* message were detected by Northern blot analysis from total RNA, and the membrane was subsequently reprobed for *GAPDH* as a loading control. Quantitation of normalized c-*myc* mRNA levels are shown as the percent relative to T cells cultured in the absence of TGFβ.

(C) HaCaT cells were transfected with siRNA-targeting E2F4 or E2F5 alone or in combination. After 60 hr, transfected and mock-transfected HaCaT cells were incubated in the presence (+) or absence (−) of TGFβ for 2 hr. Total RNA was isolated and the mRNA levels of c-*myc*, *E2f4*, and *E2f5* were detected by Northern blot and quantitated by densitometry.

(D) HaCaT cells were transfected with the indicated siRNA. Sixty hours after transfection, cells were treated with (+) or without (−) TGFβ prior to lysis and Smad2/3 immunoprecipitation. Smad-associated p107 was detected by immunoblotting.

to the established role of E2Fs as ultimate recipients of cell cycle regulatory signals that control the cdk/pRb/E2F pathway. The role elucidated here places E2Fs as cotransducers of TGFβ receptor signals that directly repress c-*myc* and contribute to inhibiting cdk. Figure 8 illustrates the relationship between these two roles of E2Fs.

E2F1, -2, and -3 are essential for cell proliferation (Wu et al., 2001b) and act as transcriptional activators of components required in S phase (Claassen and Hann, 2000; Gaubatz et al., 2000; Ren et al., 2002; Trimarchi and Lees, 2002). The activity of E2F1-3 is inhibited by binding of pRb and reactivated by cdk-mediated release from pRB. E2F4 and E2F5, on the other hand, act primarily as transcriptional repressors, and do so in association with p107 or p130. p130-E2F4/5 complexes progressively accumulate when cells undergo cdk inhibition and G1 arrest in response to mitogen deprivation (Moberg et al., 1996; Takahashi et al., 2000; Tommasi and Pfeifer, 1997; Vairo et al., 1995; Zwicker et al., 1996) or TGFβ stimulation (Iavarone and Massagué, 1999; Yagi et al., 2002). p130-E2F4/5 complexes repress a large set of cell cycle genes, thus maintaining a G0 state until cells are stimulated to reenter the division cycle (Ren et al., 2002; Takahashi et al., 2000).

The role of E2F4 and E2F5 as rapid mediators of c-*myc* repression by TGFβ described here is unrelated, both temporally and mechanistically, to their role as repressors in G0 (Figure 8). Our evidence shows that a pool of E2F4/5 and p107 is preassembled with Smad3 in the

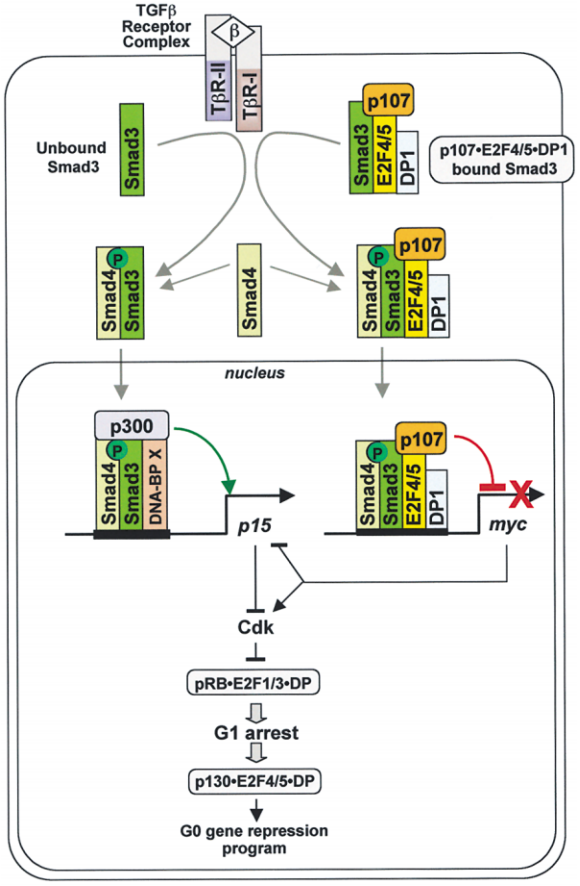

Figure 8. Schematic Representation of the TGFβ Pathway that Signals c-*myc* Repression

TGFβ regulates gene expression by a canonical mechanism involving phosphorylation of Smad3, which then binds Smad4, translocates into the nucleus, and associates with DNA binding partners and transcriptional coactivators (such as p300). This mechanism has been demonstrated for various TGFβ target genes, and in the TGFβ cytostatic program, it applies to the induction of *p15Ink4b* and *p21Cip1* cdk inhibitors. Discussions of the c-*myc* response and downstream events are included in the main body of the text. In the scheme, thin arrows represent direct effects or molecular interactions, and block arrows represent multistep processes.

cytoplasm. TGFβ receptor activation enables nuclear accumulation of this complex, incorporation of Smad4, and binding to the inhibitory element of c-*myc*. These rapid events are completed within 1 hr of TGFβ addition and can be triggered at any point during the cell cycle. That is, the rapid c-*myc* response to TGFβ mediated by the Smad-E2F4/5-p107 complex is a cell cycle-independent event contributing to, rather than deriving from, G1 arrest. The Smad-E2F4/5-p107 complex described here might target additional genes in response to TGFβ. However, transcriptomic analyses of the TGFβ response in *E2f4*- and *E2f5*-depleted HaCaT cells indicate that this group of genes is very limited (our unpublished results).

A Preassembled *myc*-Targeting Complex with Smads and E2Fs as DNA Binding Partners

In the basal state, the endogenous Smad3 protein is largely found in large complexes of previously unknown composition or function (Jayaraman and Massague, 2000). We now define one of those complexes as Smad3-p107-E2F4/5. The fact that this complex exists prior to TGFβ stimulation argues that Smad3 can be preassembled into complexes of defined gene targeting specificity. The pool of Smad3 in unstimulated cells may include additional complexes with other partners to target different sets of genes upon TGFβ stimulation. The responsiveness of a cell to TGFβ may thus be configured by preexisting Smad complexes and may be modified by conditions affecting the abundance, activity, and/or localization of Smad3 partners even before TGFβ stimulation occurs. This represents a departure from previous cases in which Smads achieve target gene specificity once they meet DNA binding cofactors in the nucleus (Chen et al., 1997; Germain et al., 2000; Hata et al., 2000; Labbé et al., 1998). It is also worth noting that both Smads and E2Fs are known to bind promiscuously or constitutively to many sites in vitro that are irrelevant or subject to considerable specific regulation in vivo. We conclude that interactions between Smad3 and E2F4/5 allow each factor to contribute to the specificity of c-*myc* TIE regulation.

Context-Dependent c-*myc* Responsiveness

Myc downregulation is an important gene response in TGFβ-induced cytostasis but is not uniformly present in cells that are growth inhibited by TGFβ (Depoortere et al., 2000; Sanchez et al., 1995) and may not be essential for cytostasis in all tissues. In fact, each of the components of the Smad3-p107-E2F4/5 complex can be genetically inactivated in the mouse without resulting in dramatic hyperproliferative disorders. In all cases, however, discreet hyperproliferative defects occur that may be lethal depending on the genetic background. *Smad3*$^{-/-}$ mice develop normally but present with accelerated keratinocyte proliferation (Ashcroft et al., 1999), and in a different genetic background, they develop metastatic colon tumors with high penetrance (Zhu et al., 1998). *p107* $^{-/-}$ mice are nearly normal in one genetic background (Sage et al., 2000) but show myeloid hyperproliferation and a shorter cell cycle in another (LeCouter et al., 1998). Mice deficient in *E2f4* or *E2f5* suffer diverse defects but are viable, whereas the combined inactivation of *E2f4* and *E2f5* leads to neonatal lethality and cell resistance to growth arrest (Gaubatz et al., 2000). Functional redundancy or compensation between family members may account for the absence of more widespread hyperproliferative phenotypes in these various cases.

In cell systems studied here, *E2f4* and *E2f5* are functionally redundant for the *c-myc* response to TGFβ. However, *p130* cannot substitute for *p107*. c-*myc* is rapidly downregulated by TGFβ in *p130*-deficient T cells but not *p107*-deficient T cells. Although E2F4 and E2F5 can bind p130 (Trimarchi and Lees, 2002), we could not detect p130 in Smad3 immunoprecipitates or on the c-*myc* promoter in ChIP assays. In the *c-myc* response to TGFβ, at least, the function of *p107* and *p130* are not redundant.

The present identification of the components of the TGFβ-dependent c-*myc* repressor complex will allow an analysis of this response and its regulation by signals

that impinge on E2F4, E2F5, or p107. It will also allow us to approach questions such as why the c-*myc* response to TGFβ is present in some but not all epithelial cells, is absent in fibroblasts that proliferate in response to TGFβ during wound healing, scar formation, and fibrosis (Roberts et al., 1986), and is selectively lost in cancer cells that gain metastatic abilities in reponse to TGFβ (Chen et al., 2001).

Experimental Procedures

Cell Culture, Transfection, and Reporter Assays

HaCaT, COS-1, and Mv1Lu-tet-p15 cells were maintained in DMEM medium and human leukemia MO-91 cells in RPMI. Media were supplemented with 10% FBS, penicillin, streptomycin, and fungizone. p15Ink4b induction in Mv1Lu-tet-p15 performed as previously described (Reynisdóttir and Massagué, 1997) using freshly replated cells. HaCaT and COS-1 transfections were performed using LipofectAMINE (Invitrogen), according to the manufacturer's instructions. Reporter assays were performed as previously described (Chen et al., 2001). For TGFβ treatment, we used 100 pM TGFβ1 (R&D Systems). To inhibit new protein synthesis, cells were treated with 10 μg/ml of cycloheximide for 1 hr.

Plasmids

To create expression vectors pHA-E2F1, pHA-E2F1(96–205), pHA-E2F4, pHA-E2F4(1–168), pHA-E2F4(169–413), pHA-E2F4(1–99), pHA-E2F5, pHA-E2F5(1–201), pHA-E2F5(202–349), pHA-E2F5(1–132), pHA-p107(1–180), p107(1–385), and p107(385–1068), the indicated coding regions were amplified from cDNA templates by polymerase chain reaction (PCR) and inserted into the *Eco*RI/*Bam*HI sites of a modified pCMV5 plasmid containing two copies of an HA epitope (Wotton et al., 1999). To generate the expression vectors pFlag-Smad3(1–145), pFlag-Smad3(146–425), pFlag-Smad3(220–425), pFlag-E2F1, pFlag-E2F4, and pFlag-E2F5, the corresponding coding regions were PCR amplified and inserted into the *Eco*RI/*Bam*HI sites of a modified pCMV5 plasmid containing a Flag epitope (Wotton et al., 1999). To construct the bacterial expression vectors pMBP-p107(1–180), pMBP-p107(181–385), pMBP-E2F4(1–168), and pMBP-E2F5(1–201), the specified coding regions were PCR amplified and cloned to the *Eco*RI/*Bam*HI sites of pMAL-c2X (NEB). To create the reporter plasmids p3xTIE-E2Fm, p3xTIE5′m1, and p3xTIE5′m2, mutant forms of the TGFβ inhibitory element (TIE) of c-*myc* (illustrated in Figure 1B) were synthesized as oligonucleotides and annealed, and three tandem copies were then inserted into the *Bam*HI site of pGL2-promoter (Promega). Reporter plasmids pFlag-F-E2Fm, pFlag-F-TIE-5′m1, and pFlag-F-TIE-5′m2 were generated by site-directed mutagenesis using primer sets containing the aforementioned mutations and pFrag-F as the template (Chen et al., 2001). Mammalian expression vectors encoding Rb, HA-p107, and HA-p130 were generously provided by A. Vidal and A. Koff (MSKCC, New York). Reporter plasmid pGL2-3XTIE has been described previously (Chen et al., 2001).

Chromatin Immunoprecipitation Assay

ChIP assays were carried out as described (Takahashi et al., 2000). Antibodies used were anti-Rb (sc-50, Santa Cruz), anti-p107 (sc-318, Santa Cruz), anti-p130 (sc-317, Santa Cruz), anti-E2F4 (sc-1082x, Santa Cruz), anti-E2F5 (sc-999, Santa Cruz), anti-Smad2/3 (Kretzschmar et al., 1999), and anti-Smad4 (Calonge and Massague, 1999). Primer set 5′-CTTTATAATGCGAGGGTCTGGAGC-3′ and 5′-GCTATGGGCAAAGTTTCGTGGATG-3′ were used to amplify a ~400 bp region of the c-*myc* promoter harboring the TIE. The primer set used to amplify the promoter region of β-*actin* was as described (Takahashi et al., 2000).

DNA Precipitation Assay

DNA precipitation assays were carried out as described previously (Chen et al., 2001). The sequences of wild-type and mutant TIE oligonucleotides are illustrated in Figure 1B. To study the Smad3-associated TIE complex, Flag-tagged immunoprecipitates were eluted with Flag-tagged peptide as described (Bonni et al., 2001), and the eluates were subjected to DNA precipitation assays.

Immunoprecipitation, Immunoblotting, and Cdk2 Kinase Assays

To detect endogenous-exogenous or exogenous-exogenous protein complexes, COS-1 cells were transfected with expression plasmid(s) encoding the indicated proteins. Thirty-six hours post-transfection, COS-1 cells were incubated for 1 hr in the presence or absence of TGFβ. Cells were lysed by sonication in HKMG buffer (Chen et al., 2001), precleared with purified mouse or rabbit IgG, and centrifuged at 13,000 × g for 30 min. Supernatants were incubated with antibodies and protein A/G beads for 3 hr at 4°C on a rotating platform. Immunoprecipitates were separated by 8%–15% SDS-PAGE and transferred to PVDF membranes (Immobilon-P, Millipore). Immunoblotting was carried out as previously described (Wotton et al., 1999). For detection of endogenous Smad3-associated proteins, 2×10^7 HaCaT cells or 2×10^8 MO-91 cells were treated with or without TGFβ for 1 hr and lysed by sonication in 3 ml of HKMG buffer. Lysates were precleared with purified rabbit IgG and centrifuged at 13,000 × g for 30 min. Immunoprecipitation and immunoblotting were performed as described above. To show the presence of Smad3-E2F4/5-p107 trimeric complex, Flag-tagged immunoprecipitates were eluted with Flag-tagged peptide as described (Bonni et al., 2001), and the eluates were subjected to a second round of immunoprecipitation followed by immunoblot analysis. Antibodies and agarose-conjugated antibodies used for the above analyses included anti-HA agarose (Sigma), anti-HA-Peroxidase (3F10, Roche), anti-Flag M2 agarose (Sigma), anti-Flag M2-peroxidase (Sigma), anti-E2F4 (Cat#05-312, Upstate Biotechnologies), anti-DP1 (05-381, Upstate Biotechnologies), anti-p107 (sc-318, Santa Cruz), anti-Rb (Cat#14001A, Pharmingen), anti-p15 (sc-612, Santa Cruz), anti-p21 (MS-891-P, NeoMarkers), anti-cdk2 (Reynisdóttir and Massagué, 1997), anti-Smad2/3 (Kretzschmar et al., 1999), and anti-Smad4 (Calonge and Massague, 1999). Purified rabbit IgG or mouse IgG was used as negative controls. For phosphatase treatment of cell extracts, lambda protein phosphatase was used as described by the manufacturer (New England Biolabs). Cdk2-associated Rb kinase activity was assayed as described previously (Reynisdóttir et al., 1995).

GST Pull-Down Assay

GST, GST-Smad3, MBP-p107(1–180), MBP-p107(181–385), MBP-E2F4(1–168), and MBP-E2F5(1–201) were expressed in *E. coli* strain BL21 and were purified as described (Truant et al., 1999). GST and GST-Smad3 (6 μg and 3 μg, respectively) were collected by glutathione beads and mixed with 1 μg of each MBP-fusion protein in HKMG buffer (Chen et al., 2001). The beads were washed and analyzed by immunoblotting.

Cell Fractionation

HaCaT cells were incubated for 1 hr in the presence or absence of TGFβ (100 pM) and collected by scraping into 1× phosphate-buffered saline (PBS). The cytoplasmic fraction was prepared using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Pierce), following the manufacturer's instructions. Following removal of the cytoplasmic fraction, the pellet was resuspended in 1.0 ml of lysis buffer (100 mM KCl, 10 mM HEPES [pH 7.9], 10% glycerol, 1 mM DTT, 5 mM $MgCl_2$, 0.1% NP40, 10 mM NaF) containing 1× protease inhibitors (Complete™ Mini, EDTA free, Roche), 1 mM sodium orthovanadate, and 1 mM β-glycerolphosphate. The samples were vortexed briefly and subjected to two rounds of sonication. Lysates were stored on ice for 40 min and vortexed vigorously every 10 min during the incubation. The nuclear fraction was isolated following centrifugation for 10 min at 14,000 rpm in a microcentrifuge. Antibodies specific to p107 (sc-318) and Lamin A/C (sc-7293; 1:500) were purchased from Santa Cruz Biotechnology. Detection of α-tubulin (T9026) was performed using antibodies obtained from Sigma, and Smad2/3-specific rabbit polyclonal antibodies have been described previously (Kretzschmar et al., 1999). DNaseI treatment of cytosolic fraction was performed using 10 U/ml of DNaseI at 4°C for 30 min. Aliquots of lysates were incubated with 1 μg of a linealized DNA to monitor the efficiency of DNaseI.

Immunofluorescence Analysis

COS-1 cells were transfected with or without FLAG-Smad3 and/or HA-p107(1–180) using Fugene 6 Transfection Reagents (Roche Molecular Biochemicals). Forty-eight hours after transfection, cells were incubated in the presence or absence of TGFβ for 1 hr. Immunofluorescence staining was carried out essentially as previously reported (Truant et al., 1999) with polyclonal rabbit anti-p107 (sc-318, Santa Cruz) and monoclonal mouse anti Smad2/3 (S66220, BD Transduction Laboratories) antibodies. Images were obtained on a Nikon Eclipse TE300 microscope.

Cell Elutriation

MO-91 cells grown to a density of 5×10^6 cells/ml were subjected to elutriation as described (Koff et al., 1992). After elutriation, cells were recovered at room temperature for 30 min and were then treated with TGFβ for 2 hr at 37°C. An aliquot of cells from each treatment was collected and subjected to flow cytometric analysis to determine the DNA content, while the rest of cells were frozen at −80°C. After the DNA content was determined, the fraction representing each cell cycle stage was selected. Lysates were then prepared from these samples and subjected to immunoblotting or immunoprecipitation.

Isolation of Primary Splenic T Cells

Spleens were isolated from wild-type, $p107^{-/-}$, and $p130^{-/-}$ males (Cobrinik et al., 1996; Lee et al., 1996) in RPMI 1640 media containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM glutamine, 0.1 mM nonessential amino acids, 5 mM HEPES, 0.05 mM β-mercaptoethanol, and 4 mM NaOH. Single-cell suspensions were prepared by mechanical disruption of the spleens through a 70 μm cell strainer (Falcon 2350) followed by centrifugation. Splenocytes were resuspended in ACK lysing buffer (2.5 mls/spleen) (Cat#10-548E; Bio-Whittaker) and incubated on ice for 5 min to lyse the red blood cells. An equal volume of RPMI 1640 media was added, and the cells were centrifuged and washed once in MACs buffer (1× PBS, 2 mM EDTA, 0.5% BSA). T cells were isolated using Thy1.2 (CD90) MicroBeads (Miltenyi Biotec) following manufacturer's instructions. The purity of selected T cell fractions was assessed by FACs analysis following staining with a FITC-conjugated hamster anti-mouse CD3 antibody (1:50 dilution; Cat#HM3401, Caltag Laboratories). For activation, Thy1.2-positive T cells were plated at a density of 2.5×10^6 cells/ml in a volume of 3 ml in a 6-well plate, which had been coated overnight with 1 μg/ml of hamster anti-mouse CD3 antibody (Cat#HM3400, Caltag Laboratories). The cells were cultured for 48 hr and then incubated for an additional 2 hr in the presence or absence of TGFβ (100 pM) prior to RNA extraction.

Small Interfering RNA (siRNA)

siRNA duplexes targeting *E2f4* and *E2f5* were prepared by annealing two pairs of 21-ribonucleotide oligonucleotides synthesized by Dharmacon Research (Lafayette, CO). The coding strands of the two siRNAs were GCGGCGGAUUUACGACAUU*TT* for *E2f4* and GUUCGU GUCGCUGCUGCAG*TT* for *E2f5*. Italics indicate deoxynucleotides. HaCaT cells were transfected with siRNA duplexes using Oligofectamine (Invitrogen). Sixty hours after transfection, cells were treated with TGFβ for 2 hr. Total RNA and cell lysates were prepared and subjected to Northern blot analysis and immunoprecipitation, respectively. Nothern blot analysis was performed as described previously (Calonge and Massague, 1999).

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